REMARKS

The above amendments to the specification are made to correct typographical and grammatical errors which occurred during the preparation of the specification and to add an Abstract of the Disclosure to the application. No new matter has been added by way of the above amendments.

If there are any minor matters precluding allowance of the application which may be resolved by a telephone discussion, the Examiner is respectfully requested to contact Mark J. Nuell, Ph.D. (Reg. No. 36,623) at (703) 205-8000.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. §§1.16 or 1.17; particularly, extension of time fees.

Respectfully submitted,

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Enclosures:
Mark-up Version Showing Changes
Abstract

MARK-UP VERSION SHOWING CHANGES

In the Specification:

The specification has been amended as follows.

Page 4

The first full paragraph (lines 10-30) was amended as follows.

In the first example a cDNA copy of a defective interfering (DI) virus variant of Sindbis virus was used to carry the CAT gene. RNA was transcribed in vitro and used to transfect avian cells and some CAT protein production could be demonstrated after infecting cells with wild-type Sindbis virus. The latter viirus provided the viral replicase for expression of the CAT construct. The inefficiency of this system depends on 1) low level of initial DI-CAT RNA transfection (0.05-0.5 % of cells) and 2) inefficient usage of the DI-CAT RNA for protein translation because of unnatural and suboptimal protein [intitation] initiation translation signals. This same system also results in packaging of some of the recombinant DI-CAT genomes into virus, particles. However, this occurs simultaneously with a very large excess of wild-type Sindbis virus production. Therefore, the usage of this mixed virus stock for CAT expression will be much hampered by the fact that most of the replication and translation activity of the cells infected with such a stock will deal with the wild-type and not with recombinant gene expression.

Page 5

The second paragraph (page 5, line 25 to page 6, line 5) was amended as follows.

Development of safe and effective vaccines against viral diseases has proven to be quite a difficult task. Although many existing vaccines have helped to combat the worldwide spread of many infectious diseases, there is still a large number of infectious agents against which effective vaccines are missing. The current procedures of preparing vaccines present several problems: (1) it is often difficult to prepare sufficiently large amounts of antigenic material; (2) In many cases there is the additional hazard that the vaccine preparation is not killed or sufficiently attenuated; (3) Effective vaccines are often hard to produce since there is a major difficulty in presenting the antigenic epitope in an immunologically active form; (4) In the case of many viruses, genetic variations in the antigenic components [results] <u>result</u> in the evolution of new strains with new serological specificities, which again creates a need for the development of new vaccines.

Page 6

The first full paragraph (lines 6-30) was amended as follows.

Two types of viral DNA vectors have been developed in order to overcome many of these problems in vaccine production. These either provide recombinant viruses or provide chimaeric viruses. The

recombinant viruses contain a wild-type virus package around a recombinant genome. These particles can be used to infect cells which then produce the antigenic protein from the recombinant genome. The chimaeric viruses also contain a recombinant genome but this specifies the production of an antigen, usually as part of a normal virus structural protein, which then will be packaged in progeny particles and e.g. exposed on the surface of the viral spike proteins. The major advantages of these [kind] kinds of virus preparations for the purpose of being used as a vaccine are 1) that they can be produced in large scale and 2) that they provide antigen in a natural form to the immunological system of the organism. Cells, which have been infected with recombinant viruses, will synthesize the exogenous antigen product, process it into peptides that then present them to T cells in the normal way. In the case of the chimaeric virus there is, in addition, an exposition of the antigen in the context of the subunits of the virus particle itself. Therefore, the chimaeric virus is also called an epitope carrier.

Page 6

The last paragraph (page 6, line 31 to page 7, line 7) was amended as follows.

The major difficulty with these [kind] kinds of vaccine preparations are, how to ensure a safe and limited replication of the particles in the host without side effects. So far, some success has

been obtained with vaccinia virus as an example of the recombinant virus approach (69) and of polio virus as an example of a chimaeric particle (70-72). As both virus variants are based on commonly used vaccine strains one might argue that they could be useful vaccine candidates also as recombinant respectively chimaeric particles (69-72). However, both virus vaccines are combined with the risk for side effects, even severe ones, and in addition these virus strains have already been used as vaccines in large parts of the population in many countries.

Page 7

The first full paragraph (lines 8-14) was amended as follows.

As is clear from the **[**afore mentioned**]** aforementioned discussion there is much need to develop improved DNA expression systems both for an easy production of important proteins or polypeptides in high yields in various kinds of animal cells and for the production of recombinant viruses or chimaeric viruses to be used as safe and efficient vaccines against various pathogenes.

The fourth full paragraph (lines 31-34) was amended as follows.

Alphavirus is a genus belonging to the family Togaviridae having single stranded RNA genomes of positive polarity enclosed in a nucleocapsid surrounded by an **[**evelope**]** envelope containing viral spike proteins.

Page 9

The second full paragraph (lines 11-24) was amended as follows.

According to a suitable embodiment of the invention such infectious particles are produced by cotransfection of animal host cells with the present RNA which lacks part of or the complete region(s) encoding the structural viral proteins together with a helper RNA molecule transcribed in vitro from a helper DNA vector comprising the SP6 promoter region, those 5′ and 3′ regions of the alphavirus cDNA which encode cis acting signals needed for RNA replication and the region encoding the viral structural proteins but lacking essentially all of the nonstructural virus proteins encoding regions including [sequenses] sequences encoding RNA signals for packaging of RNA into nucleocapsid particles, and culturing the host cells.

Page 12

The second full paragraph (lines 12-15) was amended as follows.

A further suitable transformation process is based on infection of the animal host cells with the [above mentioned] above-mentioned infectious viral particles comprising the present RNA molecule.

Page 13

The fifth full paragraph (lines 18-28) was amended as follows.

For instance, if the chimaeric virus particles containing the [afore mentioned] aforementioned conditional lethal mutation in its [s tructural] structural proteins (a defect to undergo I a certain proteolytical cleavage in host cell during morphogenesis) is used as a vaccine then [this is] such chimaeric virus particles are first activated by limited proteolytic treatment before being given to the organism so that [it] they can infect recipient cells. New chimaeric particles will be formed in cells infected with the activated virus but these will again [be of the lethal] have the conditional lethal phenotype and further spread of infection is not possible.

Page 14

The first full paragraph (lines 4-11) was amended as follows.

Still another aspect of the invention is to use a recombinant virus containing exogenous RNA encoding a polypeptide antigen for vaccination purpose or to produce antisera. In this case the recombinant virus or the conditionally lethal variant of it is used to infect cells in vivo and antigen production will take place in the [infectious] infected cells and used for antigen presentation to the immunological system.

Between the second and third full paragraphs (between lines 16 and 17), the following subheading was added.

Brief Description of the Drawings

Page 15

The first and second full paragraphs (lines 2-18) were amended as follows.

[Fig. 4 A-C] Figs 4A-4C show the construction of full-length infectious clones of SFV; Fig. 4A shows a schematic restriction map of the SFV genome; primers used for initiating cDNA synthesis are indicated as arrows, and the cDNA inserts used to assemble the final clone are [showed] shown as bars; Fig. 4B shows plasmid pPLH211, i.e. the SP6 expression vector used as carrier for the full-length infectious clone of SFV, and the resulting plasmid pSP6-SFV4; Fig. 4C shows the structure of the SP6 promoter area of the SFV clone; the stippled bars indicate the SP6 promoter sequence, and the first [necleotide] nucleotide to be transcribed is marked by an asterisk; underlined regions denote authentic SFV sequences;

[Fig. 5 shows] Figs. 5(1)-5(18) show the complete nucleotide sequence of the pSP6-SFV4 RNA transcript as DNA (U = T) and underneath the DNA sequence, the amino acid sequence of the non-structural polyprotein and the structural polyprotein;

The fourth full paragraph (lines 22-23) was amended as follows.

[Fig. 7 shows] Figs 7(1)-7(3) show the construction of the SFV expression vectors pSFV1-3 and of the Helper 1;

Page 16

The first full paragraph (lines 1-10) was amended as follows.

[Fig. 12 shows] Figs 12(1)-12(2) show in its upper part sequences encompassing the major antigenic site of SFV and the in vitro made substitutions leading to a BamHI restriction endonuclease site, sequences spanning the principal neutralizing domain of the HIV gp120 protein, and the HIV domain inserted into the SFV carrier protein E2 as a BamHI oligonucleotide; and its lower part is a schematic presentation of the SFV spike structure with blow-ups of domain 246-251 in either wild type or chimaeric form.

The third full paragraph (lines 19-35) was amended as follows.

With reference to [Fig.] Figs. 1-3, in the following the SFV and its replication are explained more in detail. In essential parts, this disclosure is true also for the other alphaviruses, such as the Sindbis virus, and many of the references cited in this connection are indeed directed to the Sindbis virus. SFV consists of an RNA-containing nucleocapsid and a surrounding membrane composed of a lipid bilayer and proteins, a regularly arranged icosahedral shell of a protein called C protein forming the capsid inside which the genomic RNA is packaged. The capsid is surrounded by the lipid bilayer that contains three proteins called E1, E2, and E3. These so-called envelope proteins are glycoproteins and their glycosylated portions are on the outside of the lipid bilayer, complexes of these

proteins forming the "spikes" that can be seen in electron micrographs to project outward from the surface of the virus.

Page 20

The last paragraph (page 6, line 33 to page 7, line 2) was amended as follows.

6. In nature SFV is of very low pathogenicity for humans. In addition, the stock virus produced in tissue culture cells is apparently apathogenic. By means of specific mutations it is possible to create conditionally lethal mutations of SFV, a feature that is of great use to uphold safety when [massproduction] mass production of virus stocks is necessary.

Page 22

The first paragraph (page 22, line 15 to page 23, line 18) was amended as follows.

3. Metabolic labeling and immunoprecipitation. Confluent monolayers of BHK cells grown in MEM supplemented with 10 mM HEPES, 2 mM glutamine, 0.2 % BSA, 100 IU/mol of penicillin and 100 µg/ml streptomycin, were infected at a multiplicity of 50 at 37°C. After 1 h p.i. the medium was replaced with fresh medium and growth continued for 3.5 h. The medium was removed and cells washed once with PBS and overlayed with methionine-free MEM containing 10 mM HEPES and 2 mM glutamine. After 30 min at 37°C the medium was replaced with the same

containing 100 μCi/ml of [^{35}S]methionine (Amersham) and the plates incubated for 10 min at 37°C. The cells were washed twice with labeling medium containing 10X excess methionine and then incubated in same medium for various times. The plates were put on ice, cells washed once with ice-cold PBS and finally lysis buffer (1 % NP-40 - 50 mM Tris-HCl, pH 7.6 - 150 mM NaCl - 2 mM EDTA) containing 10 μg/ml PMSF (phenylmethylsulfonyl fluoride) was added. Cells were scraped off the plates, and nuclei removed by centrifugation at 6,000 rpm for 5 min at 4°C in an Eppendorf centrifuge. Immunoprecipitations of proteins was performed as described (31). Briefly, antibody was added to lysate and the mixture kept on ice for 30 min. Complexes were recovered by binding to Pansorbin for 30 min on ice. Complexes were washed once with low salt buffer, once with high salt buffer, and once with 10 mM Tris-HCl, pH 7.5, before heating with gel loading buffer. To [precipitate dhfr,] immunoprecipitate particular proteins, SDS was added to 0.1 % and the mixture heated to 95°C for 2 min followed by addition of 10 volumes of lysis buffer. [Anti-E1[8.139],] Antibodies employed for the immunoprecipitation are as follows. Anti-E1 [8.139], anti-E2 [5.1] (36), and anti-C [12/2] (37) monoclonals have been described. The human transferrin receptor was precipitated with the monoclonal antibody OKT-9 in ascites fluid. This preparation was provided by Thomas Ebel at our laboratory using a corresponding hybridoma cell line obtained from ATCC (American [Typ] Type Culture Collection) No CRL 8021. Polyclonal rabbit anti-

mouse dhfr was a kind gift from E. Hurt (European Molecular Biology Laboratory, Heidelberg, FRG) and rabbit anti-lysozyme has been described (38).

Page 23

The last paragraph (page 23, last line to page 24, line 14) was amended as follows.

5. DNA procedures. Plasmids were grown in Escherichia coli DH5α (Bethesda Research Laboratories) [recA endA1 gyrA96 thi1 hsdR17 supE44 relA1 Δ(lacZYA-argF)U169 ø80dlacZΔ(M15)]. All basic DNA procedures were done essentially as described (39). DNA fragments were isolated from agarose gels by the freeze-thaw method (40) including 3 volumes of phenol during the freezing step to increase yield and purity. Fragments were purified by benzoyl-naphthoyl-DEAE (BND) cellulose (Serva Feinbiochemica, Heidelberg, FRG) chromatography (41). Plasmids used for production of infectious RNA were purified by sedimentation through 1 M NaCl followed by banding in CsCl (39). In some cases plasmids were purified by Qiagen chromatography [(Diagen] (Qiagen Gmbh, Düsseldorf, FRG).

Page 24

The first paragraph (page 24, line 15 to page 25, line 12) was amended as follows.

6. Site-directed oligonucleotide mutagenesis. For oligonucleotide mutagenesis, relevant fragments of the SFV cDNA clone were subcloned into M13mp18 or mp 19 (42) and transformed (43) into DH5αaFIQ [endA1 hsdR1 supE44 thi1 recA1 gyrA96 relA1 ø80dlacΔ(M15) Δ(lacZYAargF)U169/F'proAB lacl^q lacZΔ(M15) Tn 5] (Bethesda Research Laboratories). RF DNA from these constructs was transformed into RZ1032 (44) [Hfr KL16 dut1 ung1 thi1 relA1 supE44 zbd279:Tn10.], and virus grown in the presence of uridine to incorporate uracil residues into the viral genome. Single stranded DNA was isolated by phenol extraction from PEG precipitated phage. Oligonucleotides were synthesized on an Applied Biosystems 380B synthesizer and purified by gel filtration over NAP-5 columns (Pharmacia). The oligonucleotides 5'-CGGCCAGTGAATTCTGATTGGATCCCGGGTAATTAATTGAATTACATCCCTACGCAAACG, (SEQ ID. NO.:13) 5'-GCGCACTATTATAGCACCGGCTCCCGGGTAATTAATTGACGCAAACGTTTTA-CGGCCGCCGG (SEQ. ID. NO.:14) and 5'-GCGCACTATTATAGCACCATG-GATCCGGGTAATTAATTGACGTTTTACGGCCGCCGGTGGCG (SEQ. ID. NO.: 15) were used to insert the new linker sites (BamHI-SmaI-XmaI] into the SFV cDNA clone. The oligonucleotides 5'-CGGCGGTCCTAGATTGGTGCG (SEQ. ID. NO.: 16) and 5'-CGCGGGCGCCACCGGCGGCCG (SEQ. ID. NO.:17) were used as sequencing primers (SP1 and SP2) up- and downstream of the polylinker site. Phosphorylated oligonucleotides were used in mutagenesis with Sequenase [(Unites] (United States Biochemicals, Cleveland, Ohio) as described earlier (44, 45). In vitro made RF forms were transformed into DH5αF'IQ and the resulting phage isolates analyzed for the

presence of correct mutations by dideoxy sequencing according to the USB protocol for using Sequenase. Finally, mutant fragments were reinserted into the full-length SFV cDNA clone. Again, the presence of the appropriate mutations was verified by sequencing from the plasmid DNA. Deletion of the 6K region has been described elsewhere.

Page 26

The last paragraph (page 25, line 33 to page 27, line 5) was amended as follows.

In this example a full-length SFV cDNA clone is prepared and placed in a plasmid containing the SP6 RNA polymerase promoter to allow in vitro [trancription] transcription of full-length and infectious transcripts. This plasmid which is designated pSP6-SFV4 has been deposited on 28 NOV 1991 at PHLS Centre for Applied Microbiology & Research European Collection of Animal Cell Cultures, Porton Down, Salisbury, Wiltshire, [U.K:,] U.K., and given the provisional accession number 91112826.

Page 27

The first and second paragraphs (page 27, line 6 to page 29, line 22) were amended as follows.

As illustrated in Fig. 4A-C the strategy for construction the SFV clone was to prime cDNA synthesis on several positions along the template RNA downstream of suitable restriction endonuclease sites

defined by the known nucleotide sequence of the SFV RNA molecule. Virus RNA was isolated by phenol-chloroform extraction from purified virus (obtainable among others from the Arbovirus collection in Yale University, New Haven, USA) and used as template for cDNA synthesis as previously described (50). First strand synthesis was primed at three positions, using 5'-TTTCTCGTAGTTCTCCTCGTC (SEQ. ID. NO.:18) as primer-1 (SFV coordinate 2042-2062) and 5'-GTTATCCCAGTGGTTGTTCTCGTAATA (SEQ. ID. NO.:19) as primer-2 (SFV coordinate 3323-3349) and an oligo-dT_{12-18} as primer -3 (3' end of SFV) Fig. 4A).

Second strand synthesis was preceded by hybridization of the oligonucleotide 5'-ATGGCGGATGTGTGACATACACGACGCC [(identical] (SEQ. ID. NO.:20, identical to the 28 first bases of the genome sequence of SFV) to the first strand cDNA. After completion of second strand synthesis cDNA was trimmed and in all cases except in the case of the primer-1 reaction, the double-stranded adaptor 5'-AATTCAAGCTTGCGGCCGCACTAGT / GTTCGAACGCCGGCGTGATCA-3' (SEQ. ID. NO.:21) (5'-sticky-EcoRI-HindIII-NotI-XmaIII-SpeI-blunt-3') was added and the [cDNa] cDNA cloned into EcoRl cleaved pTZ18R (Pharmacia, Sweden) as described (51). The cloning of the 5' end region was done in a different way. Since SFV contains a HindIII site at position 1947, cDNA primed with primer-1 should contain this area and therefore HindIII could be used to define the 3' end of that cDNA. To obtain a restriction site at the very 5' end of the SFV, cDNA was

cloned into SmaI-HindIII cut pGEM1 (Promega Biotec., Madison, WI). Since the SFV genome starts with the sequence 5'-ATGG, ligation of this onto the blunt CCC-3' end of the SmaI site created an NcoI site C'CATGG. Although the SFV sequence contains 3 NcoI sites, none of these are within the region preceding the HindIII site, and thus these 5' end clones could be further subcloned as NcoI-HindIII fragments into a vector especially designed for this purpose (see below). The original cDNA clones in pGEM1 were screened by restriction analysis and all containing inserts bigger than 1500 bp were selected for further characterization by sequencing directly from the plasmid into both ends of the insert, using SP6 or T7 sequencing primers. The SFV 5'-end clones in pTZ18R were sequenced using lac sequencing primers. To drive in vitro synthesis of SFV RNA the SP6 promoter was used. Cloning of the SFV 5' end in front of this promoter without adding too many foreign nucleotides required that a derivative of pGEM1 had to be constructed. Hence, pGEM1 was opened at EcoR1 and Bal31 deletions were created, the DNA blunted with T4 DNA polymerase and an NcoI oligonucleotide (5'-GCCATGGC[)], SEQ. ID. NO.:22) added. The clones obtained were screened by colony hybridization (39) with the oligonucleotide 5'-GGTGACACTATAGCCATGGC (SEQ ID NO.:23) designed to pick up (at suitable stringency) the variants that had the NcoI sequence immediately at the transcription initiation site of the SP6 promoter (G underlined). Since the Bal31 deletion had removed all restriction sites of the multicloning site

of the original plasmid, these were restored by cloning a PvuI-NcoI fragment from the new variant into another variant of pGEM1 (pDH101) that had an NcoI site inserted at its HindIII position in the polylinker. This created the plasmid pDH201. Finally, the adaptor used for cloning the SFV cDNA was inserted into pDH201 between the EcoRI and PvuII sites to create plasmid pPLH211 (Fig. 4B). This plasmid was then used as recipient for SFV cDNA fragments in the assembly of the full-length clone by combining independent overlapping subclones using these sites. The fragments and the relevant restriction sites used to assemble the full-length clone, pSP6-SFV4, are depicted in (Fig. 4A). For the 5'-end, the selected fragment contained the proper SFV sequence 5'-ATGG, with one additional G-residue in front. When this G-residue was removed it reduced transcription efficiency from SP6 but did not affect infectivity of the in vitro made RNA. Thus, the clone used for all subsequent work contains the G-residue at the 5' end. For the 3'-end of the clone, a cDNA fragment containing 69 A-residues was selected. By inclusion of the unique SpeI site at the 3'-end of the [cDNa,] cDNA, the plasmid can be linearized to allow for runoff transcription in vitro giving RNA-carrying 70 A-residues. Fig. 4C shows the 5' and 3' border sequences of the SFV cDNA clone. The general outline how to obtain and demonstrate infectivity of the full-length SFV RNA is depicted in Fig. 6. The complete nucleotide sequence of the pSP6-SFV4

SP6 transcript together with the amino acid sequences of the nonstructural and the structural polyproteins is shown in Fig. 5.

Page 30

The second full paragraph (lines 9-20) was amended as follows.

The cDNA clone coding for the complete genome of SFV obtained in Example 1 was used to construct [a] an SFV DNA expression vector by deletion of the coding region of the 26S structural genes to make way for heterologous inserts. However, the nonstructural coding region, which is required for the production of the nsP1-4 replicase complex is preserved. RNA replication is dependent on short 5' (nt 1-247) (53, 54, 55) and 3' (nt 11423-11441) sequence elements (56, 57), and therefore, also these had to be included in the vector construct, as had the 26S promoter just upstream of the C gene (17, 18).

The third paragraph (page 30, line 21 to page 31, line 5) was amended as follows.

As is shown in Fig. 7, first, the XbaI (6640)-NsiI (8927) fragment from the SFV cDNA clone pSP6-SFV4 from Example 1 was cloned into pGEM7Zf(+) (Promega Corp., [Wl,] WI, USA) (Step A). From the resulting plasmid, pGEM7Zf(+)-SFV, the EcoRI fragment (SFV coordinates 7391 and 88746) was cloned into M13mp19 to insert a BamHI - XmaI - SmaI polylinker sequence immediately downstream from the 26S promoter site using site-directed mutagenesis (step B). Once the

correct mutants had been [verfied] verified by sequencing from M13 ssDNA (single stranded), the EcoRI fragments were reinserted into pGEM7Zf(+)-SFV (step C) and then cloned back as XbaI-NsΛ fragments into pSP6-SFV4 (step D). To delete the major part of the cDNA region coding for the structural proteins of SFV, these plasmids were then cut with AsuII (7783) and NdeI (11033), blunted using Klenow fragment in the presence of all four nucleotides, and religated to create the final vectors designated pSFV1, pSFV2 and pSFV3, respectively (step E). The vectors retain the promoter region of the 26S subgenomic RNA and the last 49 amino acids of the E1 protein as well as the complete non-coding 3′ end of the SFV genome.

Page 31

The first full paragraph (lines 6-27) was amended as follows.

In the vectors the subgenomic (26S) protein coding portion has been replaced with a polylinker sequence allowing the insertional cloning of foreign cDNA sequences under the 26S promoter. As is shown in Fig. 8 these three vectors have the same basic cassette inserted downstream from the 26S promoter, i.e. a polylinker (BmHI-SmaI-XmaI) followed by a translational [stop-codons] stop codon in all three reading frames. The vectors differ as to the position where the polylinker cassette has been inserted. In pSFV1 the cassette is situated 31 bases downstream of the 26S transcription initiation site. The initiation [motive] motif of the capsid gene translation is

identical to the consensus sequence (58). Therefore, this [motive] motif has been provided for in pSFV2, where it is placed immediately after the [motive] motif of the capsid gene. Finally, pSFV3 has the cassette placed immediately after the initiation codon (AUG) of the capsid gene. Sequencing primers (SP) needed for checking both ends of an insert have been designed to hybridize either to the 26S promoter region (SP1), or to the region following the stop codon cassette (SP2).

Page 32

The last paragraph (page 32, line 31 to page 33, line 3) was amended as follows.

To package the RNA a region at the end of nsP1 is required, an area which has been shown to bind capsid protein (57, 59). Since the Helper lacks this region, RNA derived from this vector will not be packaged and hence, transfections with recombinant and Helper produces only virus particles that carry recombinant-derived RNA. It follows that these viruses cannot be passaged further and thus provide a one-step virus stock. The advantage is that infections with these particles will not produce any viral structural proteins.

Page 33

The last paragraph (lines 33-37) was amended as follows.

To create a unique restriction endonuclease site that would allow specific insertion of foreign epitopes into the E2 portion of the SFV genome, a BamHI site was inserted by site directed mutagenesis using the oligonucleotide [5'-GATCGGCCTAGGAGCCGAGAGCCC] 5'-GATCGGCCTAGGAGCCGAGAGCCC-3', SEQ. ID. NO.:24).

Page 34

The last paragraph (page 34, line 18 to page 35, line 7) was amended as follows.

Once activated the SFV variant will enter cells normally through the endocytic pathway and start infection. Viral proteins will be made and budding takes place at the plasma membrane. However, all virus particles produced will be of inactive form and the infection will thus cease after one round of [replication] infection. The reason for the block in infection proficiency is a mutation which has been introduced by site directed mutagenesis into the cleavage site of p62. This arginine to leucine substitution (at amino acid [postion] position 66 of the E3 portion of the p62 protein) changes the consensus features of the cleavage site so that it will not be recognized by the host cell proteinase that normally cleaves the p62 protein to the E2 and E3 polypeptides during transport to the cell surface. Instead, only exogenously added trypsin will be able to perform this cleavage, which in this case occurs at the arginine residue 65 immediately preceding the original cleavage site. As this

cleavage regulates the activation of the entry function potential of the virus by controlling the binding of the entry spike subunit, the virus particle carrying only uncleaved p62 will be completely unable to enter new host cells.

Page 35

The third full paragraph (lines 16-18) was amended as follows.

BHK cells were transfected with the above SFV RNA molecules by electroporation and optimal conditions were determined by varying parameters like temperature, voltage, capacitance, and number of pulses. Optimal transfection was obtained by 2 consecutive pulses of 1.5 kV at 25 μF, under which negligible amounts of cells were killed. It was found that it was better to keep the cells at room [tempeature] temperature than at 0°C during the whole procedure. Transfection by electroporation was also measured as a function of input RNA. As expected, an increase in transfection frequency was not linearly dependent on RNA concentration, and about 2 μg of cRNA were needed to obtain 100 % transfection.

Page 36

The first paragraph (page 36, line 11 to page 37, line 5) was amended as follows.

To study the expression of the heterologous proteins, in vitro-made RNA of the dhfr and TR constructs was electroporated into BHK

cells. RNA of wild type SFV was used as control. At different time points post electroporation (p.e.) cells were pulse-labeled for 10 min followed by a 10 min chase, whereafter the lysates were analyzed by gel electrophoresis and autoradiography. The results are shown in Figure 11. More specifically, BHK cells were transfected with RNAs of wild type SFV, pSFV1-dhfr, and pSFV1-TR, pulse-labeled at 3, 6, 9, 12, 15 and 24 h p.e. Equal amounts of lysate were run on a 12 % gel. The 9 h sample was also used in immunoprecipitation (IP) of the SFV, the dhfr and the transferrin receptor proteins. Cells transfected with pSFV1-lysozyme were pulse-labeled at 9 h p.e. and then chased for the times (hours) indicated. An equal portion of lysate or medium was loaded on the [13,5] 13.5 % gel. IP represents immunoprecipitation from the 1 h chase lysate sample. The U-lane is lysate of labeled but untransfected cells. At 3 h [p.e.hardly] p.e. hardly any exogenous proteins were made, since the incoming RNA starts with minus strand synthesis which does not peak until about 4-5 h p.e. (5). At this time point, almost all labeled proteins were of [hos] host origin. In contrast, at 6 h p.e. the exogenous proteins were synthesized with great efficiency, and severe inhibition of host protein synthesis was evident. This was even more striking at 9 h p.e., when maximum shut down of host protein synthesis had been reached. Efficient production of the heteroloqous proteins continued up to 24 h p.e., after which production slowed down (data not shown), indicating that the cells had entered a stationary phase.

Page 37

The third paragraph (page 37, line 16 to page 37, line 5) was amended as follows.

In vitro-made RNA of pSFV1-TR was mixed with Helper RNA at different ratios and these mixtures were cotransfected into BHK cells. Cells were grown for 24 h after which the culture medium was collected and the virus particles pelleted by ultracentrifugation. The number of infectious units (i.u.) was determined by immunofluorescence. It was found that a 1:1 ratio of Helper and recombinant most efficiently produced infectious particles, and on the average 5 x 10^6 cells yielded 2.5 x 10^9 i.u. The infectivity of the virus stock was tested by infecting BHK cells at different multiplicities of infection (m.o.i.). In Fig. 11 the results for expression of human transferrin receptor in BHK cells after infection by such in vivo packaged particles carrying pSFV1-TR recombinant RNA is shown to the lower right. 200 µl of virus diluted in MEM (including ~~[0,5%]~~ 0.5% BAS and 2 mM glutamine) was overlaid on cells to give m.o.i. values ranging from 5 to 0.005. After 1 h at 37°C, complete BHK medium was added and growth continued for 9 h, at which time a 10 min pulse (100 µCi ^{35}S-methionine/ml) and 10 min chase was performed, and the cells dissolved in lysis buffer. 10 µl out of the 300 µl lysate (corresponding to 30,000 cells) was run on the 10 % gel, and the dried gel was exposed for 2 h at -70°C. Due to the high

expression level, only 3,000 cells are needed to obtain a distinct band on the autoradiograph with an [over night] overnight exposure.

Page 38

The first full paragraph (line 6-12) was amended as follows.

Thus, it was found that efficient protein production and concomitant [hos] host protein shut-off occurred at about 1 i.u. per cell. Since one SFV infected cell produces on the average 10^8 capsid protein molecules, it follows that a virus stock produced from a single electroporation can be used to produce 10^{17} protein molecules equaling about 50 mg of protein.

The fifth paragraph (page 38, line 33 to page 39, line 3) was amended as follows.

(3) The recombinant virus can be used to infect the recombinant genome in a "natural" and [nonleakey] non-leakey way into a large variety of cells including insect and most higher euoaryotic cell types. Such a wide host range is very useful for an [expressions] expression system especially when cell-type-specific posttranslational modification reactions are required for the activity of the expressed protein.

Page 39

The first full paragraph (line 4-15) was amended as follows.

(4) The level of protein expression obtained is extremely high, the level corresponding to those of the viral proteins during infection. There is also a host cell protein shut-off which makes it possible to follow the foreign proteins clearly in cell lysates without the need for antibody mediated antigen concentration. This will facilitate DNA expression experiments in cell biology considerably. Furthermore, problems of interference by the endogenous [counter part] counterpart to an expressed protein (i.e. homo-oligomerization reactions) can be avoided.

The last full paragraph (line 19-35) was amended as follows.

A very important example where vaccine development is of the utmost importance concerns the acquired immunodeficiency syndrome (AIDS) caused by the human immunodeficiency virus HIV-1 (66, 67). [Sofar,] So far, all attempts to produce an efficient vaccine against HIV-1 have failed, although there was a very recent report that vaccination with disrupted SIV-1 (Simian immunodeficiency virus) to a certain extent may give protection against infections of that virus (68). However, development of safe and effective vaccine against HIV-1 will be very difficult due to the biological properties of the virus. In the present [example] example one epitope of HIV-1 was inserted into an antigenic domain of the E2 protein of SFV. The

epitope used is located in glycoprotein gp120 of HIV-1, spanning amino acids 309-325. This forms the variable loop of HIV-1 and is situated immediately after an N-glycosylated site.

The last paragraph (page 39, line 36 to page 40, line 15) was amended as follows.

A chimaera was constructed where the 309-325 epitope of HIV was inserted into the BamHI site using cassette insertion of ready-made oligonucleotides encoding the HIV epitope. The required base substitutions at the BamHI site did not lead to any amino acid changes in the vector, although two amino acids (Asp and Glu) changed places. This change did not have any deleterious effect since in [vitro made] vitro-made vector RNA induced cell infection with wild type efficiency. Fig. 12 shows the sequences in the area of interest in the epitope carrier. In preliminary experiments, it has been shown that chimaeric proteins were produced. The proteins can be immunoprecipitated with anti-HIV antibodies. It is to be expected that these are also used for production of chimaeric virus particles that can be used for vaccine preparation against HIV. Such particles are shown in Fig. 12, lower part.

The first full paragraph (Reference No. 1) was amended as follows.

1) Bishop, D.H.L. (1990). Gene expression using insect cells and viruses. In [current] Current Opinion in Biotechnology, Vol. 1, Rosenberg, M., and Moss, B., eds. (London: Current Opinion Ltd.), pp. 62-67.

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The sixth paragraph (Reference No. 36) was amended as follows.

36) Boere, W.A.M., Harmsen, T., Vinje, J., Benaissa-Trouw, B.J., Kraaijeeveld, C.A., and Snippe[.], H. (1984). Identification of distinct antigenic determinants on Semliki Forest virus by using monoclonal antibodies with different antiviral activities. J. Virol. 52, 575-582.

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The third full paragraph (Reference No. 54) was amended as follows.

54) Niesters, H.G.M., and Strauss, J.H. (1990b). Mutagenesis of the conserved [51-nucleotide] 5'-nucleotide region of Sindbis virus. J. Virol. 64, 1639-1647.

An abstract is added to the application.